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Isolated root caps, border cells, and mucilage from host roots stimulate hyphal branching of the arbuscular mycorrhizal fungus, *Gigaspora gigantea**

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Unlike previous reports that have shown that water soluble and volatile compounds from roots or root exudates play an important role in precolonization events during arbuscular mycorrhizal (AM) fungus-host root interactions (Bécard & Piché 1989, Giovannetti *et al*. 1993), the results shown here deal with particulate and viscous fractions isolated from host roots. Root caps and a slow sedimenting particulate fraction (SSPF) were rapidly isolated and separated from Ri T-DNA transformed carrot roots (*D. carota*) grown in liquid culture. In addition, border cells (BC) and mucilage were isolated from aseptically grown corn seedlings (*Zea mays*). Root caps, SSPF (composed mainly of small root cap fragments and some BCs), BCs, and mucilage all had an associated AM fungus hyphal branching stimulator. Root caps stored for 5 d at 4 °C appeared to either synthesize or slowly release the branching stimulator. Also, isolated root caps from roots grown in the absence of P contained more branch stimulating activity than those isolated from roots grown in the presence of P. Although the branching stimulation activity in particulate fractions was low compared to that of the exudate, the particulate fractions can stick to the root surface at considerable distances from the root tip. This may be significant during the infection and colonization of host roots at sites far removed from the primary location of exudation.

INTRODUCTION

Considerable work has been done on the physical and structural properties of root caps, sloughed root cap cells called border cells (BC), and root mucilage. The physical properties and role of mucilage in maintaining root-soil contact in the rhizosphere has been studied (Read, Gregory & Bell 1999) and recent work has indicated that mucilage can be used by rhizosphere bacteria as a sole carbon source (Knee *et al*. 2001). Likewise, functions and roles of root caps (Rougier 1981) and BC have also been proposed (Hawes *et al*. 1998). Although BC have been shown to be a substrate for ectomycorrhizal fungal mantle development (Hawes *et al*. 1998), a role for these cells in endomycorrhizal fungal-host root interactions has not been demonstrated. However, a recent report showed a correlation between AM fungal colonization and BC production (Niemira, Safir & Hawes 1996). In general, plant families that were high in BC production were more readily colonized by AM fungi and families that were low in BC production were less likely to be colonized (Niemira *et al*. 1996). One possible explanation for this observation is that border cells influence the growth and development of mycorrhizal fungi. The goal of this research was to determine if root caps, border cells, and/or root mucilage contain hyphal branching stimulators since hyphal branching is the first morphogenetically identifiable interaction between a host root and AM fungus and precedes appressorium formation (Giovannetti *et al*. 1993).

MATERIALS AND METHODS

Root cultures

Ri T-DNA transformed carrot root (*Daucus carota*) cultures were maintained in Petri dishes containing M medium (minimal medium) solidified with 0.2 % gellan gum (Bécard & Fortin 1988, Bécard & Piché 1992). To grow roots in liquid culture, segments of carrot roots were aseptically transferred from the solid medium into a 500 ml Erlenmeyer flask containing 250 ml of

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sterilized M medium without gellan. Roots were grown for 14 d at 24 °C in a shaking water bath and after this time, the liquid medium was replaced with fresh M medium. After an additional 14 d, the liquid medium was harvested. In some cases, after 28 d growth, the liquid was replaced with fresh sterilized M medium with (35 μM Pi) or without phosphorus and grown for 7 d more. The fresh weight of the roots averaged 15–20 g per flask at the end of the normal growth period.

Isolation of root caps and a particulate fraction from carrot roots

To harvest the root caps, the liquid culture was decanted over a sterilized Buchner funnel containing a double layer of Whatman No. 4 filter paper. The root mass remained inside the Erlenmeyer flask and was rinsed twice with sterile water to increase the yield of root caps. The root caps were trapped on the filter paper and any intact pieces of roots trapped on the filter were then removed with a sterile forceps. Small pieces of gellan from when the roots were originally transferred from solid medium to liquid were also trapped on the filter. To specifically clean up the root cap fraction, the material trapped on the filter paper was washed off with sterile water, transferred to a cone shaped centrifuge tube, pelleted at 3000 rpm for two minutes, and resuspended in 15 ml of sterile 10 mM citrate buffer at pH 6.0 for 30 min. The tube was vortexed frequently to insure that all gellan particles were dissolved (Doner & Bécard 1991). The tube was centrifuged as previously, and the pellet resuspended in 15 ml of sterile water and centrifuged. This step was repeated twice to remove the citrate buffer and all other soluble components. After the last wash, the particulate matter was vortexed in 5 ml of sterile water and stored on ice for 1 h. The root caps readily sedimented to the bottom of the tube and a second slower sedimenting particulate fraction (SSPF) settled on top of the root caps. This particulate fraction was gently removed from the root caps and the root caps were resuspended in 15 ml of sterile water. This process was repeated 3 more times and the SSPFs were combined and kept separated from the root caps.

Mucilage and border cells from aseptically germinated corn roots

Corn seeds, *Zea mays* (hybrid sweet corn, cv. 'Silver Queen'), were initially washed with cold water to remove the fungicide from the treated seeds. The washed seeds then were surface sterilized by stirring in 30 % H_2O_2 for 15 min and then rinsed three times with sterile deionized-distilled water. The seeds then were soaked in 70 % ethanol for 3 min and placed in Petri dishes containing M medium and allowed to germinate and grow for 3–5 d. At this time, any contaminated seed was discarded. Root tips were dipped in sterile water for 30 s to release the border cells (Hawes *et al*. 1998). The mucilage containing border cells then was removed from the roots with fine, aseptic, pipet tips and used directly in the bioassay. Mucilage was separated from the BC by filtering through a 0.45 μm nylon filter (Read *et al*. 1999) which trapped the BC and allowed us to test purified mucilage in the bioassay. Mucilage containing border cells was analysed for total sugar content before and after filtration using the phenol-sulphuric acid method (DuBois *et al*. 1956).

Bioassay for detecting fungal branching stimulators

Spores of *Gigaspora gigantea* were produced in pot cultures in a greenhouse with *Paspalum notatum* as a host. Voucher samples are preserved in BPI. Spores were collected, isolated, and sterilized as described earlier (Bécard & Piché 1992) and then stored at 4 ° until needed. To prepare the spores for germination, individual spores were inserted into gelled M medium and incubated in a 2 % a CO_2 atmosphere at 32 °. When a germ tube appeared, typically after 2–4 d, the spore was transferred within a core of the medium to a fresh Petri plate of M medium and grown for 3 d more at 32 ° and 2 % CO_2.

The bioassay used here was developed for soluble exudate components (Nagahashi & Douds 1999) and was modified to test particulate or viscous fractions. Two large holes were placed in the gellan about 2–3 mm from a growing hyphal tip using sterile pipet tips (200 μl, automatic Gilson-Rainin Pipetman) cut back to produce a hole 3 mm diam. 40 μl of either supernatant, root caps, the SSPF, mucilage, or border cells with mucilage were applied to each pair of holes with 20 μl into each hole. For corn roots, all of the mucilage with border cells that could be removed from a single root tip was added to the two large holes near a growing hyphal tip. Treated spores were returned to the 32 °, 2 % CO_2 atmosphere and the number of hyphal branches were counted 16–20 h later at 20× or 50× with a stereomicroscope.

Micrographs and figures of isolated root caps, the SSPF, and intact roots of carrots

Pictures of the hyphal branching of *Gigaspora gigantea* spores induced by root caps and the SSPF were taken after the plate was stained with trypan blue. Micrographs of the purified root cap fraction, SSPF, and intact carrot roots were taken without staining. A Leica MZ FLIII stereomicroscope (Leica Microsystems, Bannockburn, IL) mounted with a Leica DC 200 digital camera was used to take pictures. To show an overview of the effect of root caps or the SSPF on the induction of hyphal branching, tracings were made on the Petri dish since the overall branching pattern could not be represented in a single micrograph.

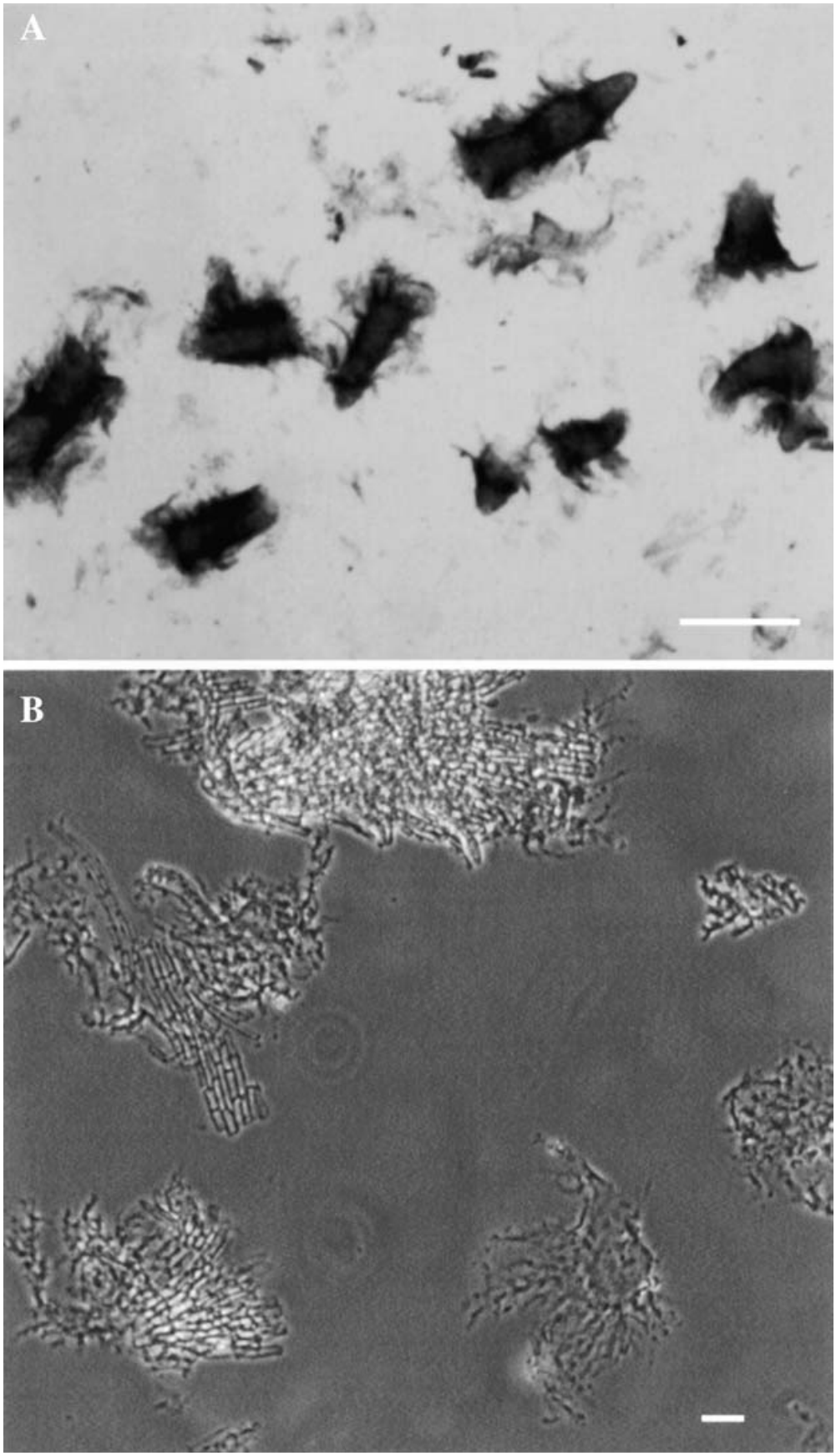

Fig. 1. Photomicrograph of root caps isolated from Ri T-DNA transformed carrot roots grown in liquid culture. (*A*) Lower magnification (marker = 225 μm) shows that the root caps are readily distinguished and are identical in morphology to root caps still attached to intact roots (*cfr* Fig. 2). (*B*) Higher magnification (marker = 75 μm) under phase contrast shows individual cells of the root caps.

RESULTS

A method involving gravity sedimentation was developed to isolate and purify root caps from roots growing in liquid medium. Fig. 1A shows a low magnification micrograph of the isolated root caps that were purified by this technique. The isolated root caps were identical in morphology to root caps of intact carrot roots growing in culture (Fig. 2). Phase contrast microscopy showed the outline of individual root cap cells (Fig. 1B). The root caps isolated from roots grown in normal M (which contains 35 μM P) medium (Bécard & Fortin 1988) stimulated hyphal branching of germinated spores of *Gigaspora gigantea* (Table 1). A cold storage treatment of purified root caps was performed to determine if the hyphal branching stimulators could readily be solubilized from the root caps. The root caps were stored in sterile water at 4 ° up to 5 d. At every test time, the test tubes were vortexed and then centrifuged to pellet the root caps, and 40 μl of supernatant was removed for the assay. 40 μl of sterile water was used to replace the aliquot and the sample again was vortexed and 40 μl of the root cap suspension was then assayed. Because the hyphal branching stimulation was increasing in the supernatant after 5 d storage (Table 1), it appeared that the branching stimulator was simply diffusing from the root caps. However, after washing the root caps one time in sterile water, testing the root cap suspension, and then centrifuging to test the clear supernatant, it was apparent that the root caps still had the same level of activity as the 0 d root caps (Table 1). The branching was noticeable after the plate was incubated for 8 h at 32 ° in a 2% CO_2 atmosphere but was most prevalent after 16–20 h. The patterns traced on the Petri plate, as depicted in Fig. 3C, show an overview of the induced morphogenetic change of germinated spores in the presence and absence of root caps. Fig. 4 shows a higher magnification of the actual root cap-induced hyphal branching.

Root caps isolated from roots grown in the absence of P for 7 d (Table 2) also readily induced hyphal branching of *G. gigantea*. The root caps were suspended in a volume of sterile water that was in direct proportion to the fresh weight of the roots from which they were harvested (1 ml sterile water : 5 g F.W.). When suspended in this fashion and directly compared, the root caps from roots grown in minus P contained more activity than root caps isolated from roots grown in normal P medium (*cfr* day 0 in Tables 1–2).

The SSPF also contained a component that stimulated hyphal branches. This particulate fraction settled by gravity on top of the root caps and microscopic examination showed that it was mainly composed of small fragments of root caps although individual BCs could be seen (Fig. 5). The presence of mucilage was not conclusively shown because what appeared to be mucilage-like turned out to be small pieces of gellan as seen before citrate buffer treatment but was dissolved with the chelator treatment (data not shown). When the isolated root caps and the SSPF from the same preparation were suspended in an identical volume and directly compared, it was apparent that the intact root cap fraction was most active (Table 2, Fig. 3).

To conclusively demonstrate that BCs and/or mucilage can stimulate hyphal branching of *G. gigantea*, carrot roots grown in liquid culture were not used because in liquid culture, the isolated BCs were heavily contaminated with small fragments of root caps. In addition, it was impossible to readily isolate BC from the small fibrous carrot roots grown on solid support medium. Instead, aseptically grown corn seedlings were used since these large roots were easy to handle and have been shown to readily produce border cells when grown on sterile filter paper placed on top of agar in Petri dishes (Hawes *et al.* 1998). The mucilage containing the border cells from an individual corn root tip was removed and applied directly near a growing hyphal tip. The data in Table 3 was expressed as the number of branches stimulated by the presence of border cells with mucilage from one corn root tip. Filtration of the mucilage-border cell mixture through a 0.45 μm filter before the bioassay, indicated that most of the branching stimulating activity was removed by the sieve since the purified mucilage typically gave only a small amount of branching stimulation (Table 3). In order to push the mucilage through the filter, the isolated BCs plus mucilage was diluted 1 : 1 with sterile water to increase the volume before filtering. This meant that the results of the bioassay with the mucilage (Table 3) was approximately one half of the actual value. This syringe-filtering system to purify the mucilage was reported earlier for corn roots (Read *et al.* 1999). To confirm that mucilage passes through the filter, the BC plus mucilage fraction was analysed for total sugar content before and after filtration through the 0.45 μm filter. The phenol sulphuric acid method showed that mucilage readily passed through the filter (84% of the total sugar was recovered) and the sugar that was retained was likely due to the BC and its cellular contents as well as its cell walls. The results from these experiments clearly indicated that the branching stimulator activity of the BC plus mucilage fraction was mainly associated with the BC.

A dose dependence response was determined for BC by isolating in water, vortexing, quickly taking aliquots and either drying or bioassaying them. The number of branches induced was linear with respect to the dry weight of the BC preparation (Fig. 6A). Similarly, a dose dependent response for isolated root caps was performed (Fig. 6B). A dose dependence for the exudate components has already been demonstrated (Nagahashi & Douds 2000), and we have determined the relationship between exudate dry weight and branching response. These data allow us to compare the relative activities of fractions stimulating hyphal branching. A plot showing the dry weight of various fractions needed to stimulate 10 hyphal branches (the 'standard effect') showed the exudate was the most

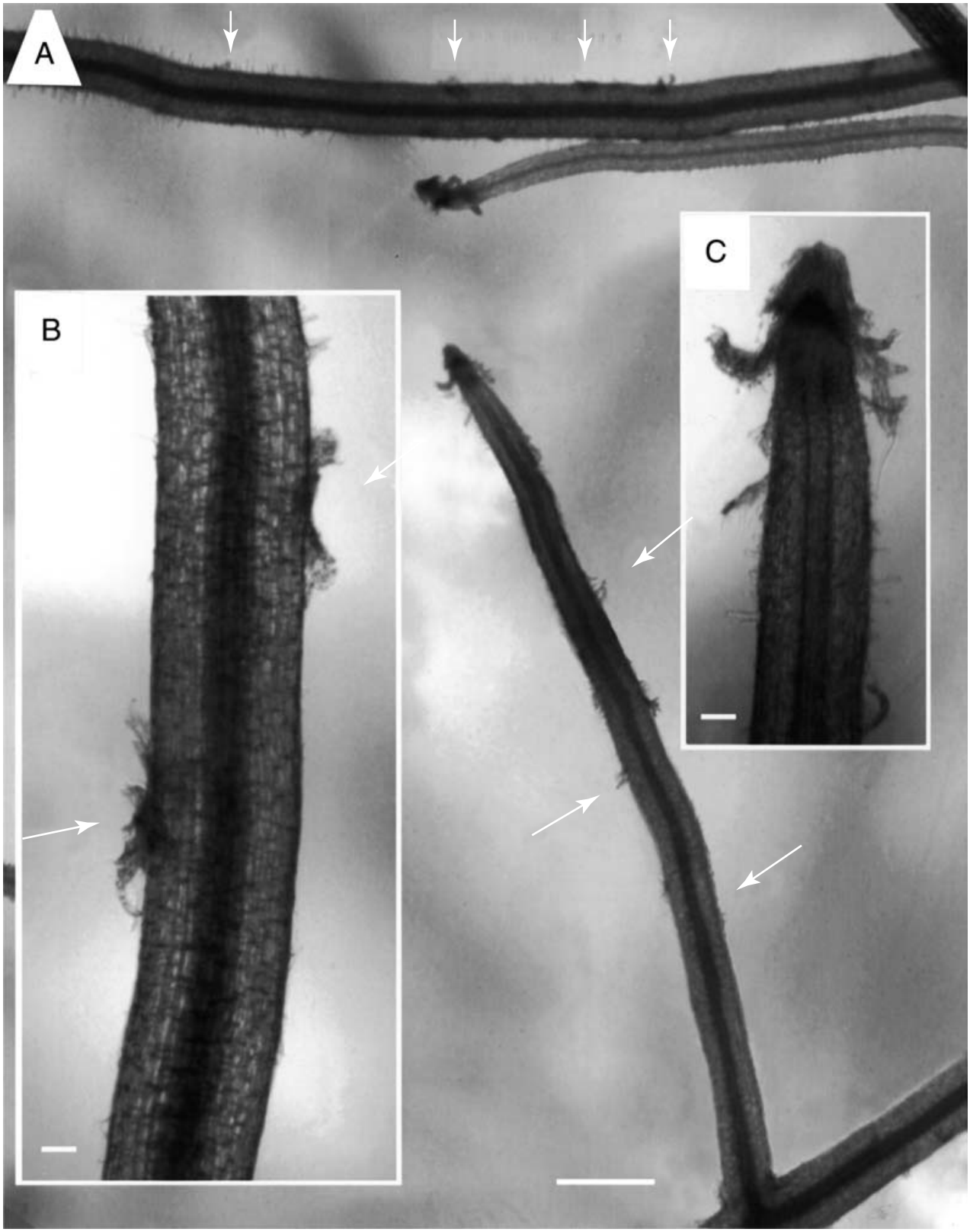

Fig. 2. Micrographs of cultured carrot roots grown on a solid support (gellan). (*A*) Micrograph showing the roots and root caps of intact roots. The white arrows mark fragments of the root cap or border cells adhering to the root surface at various distances from the root tip. Marker = 450 μm. (*B*) Higher magnification of the attachment of root caps or border cells to the root surface 10 cm behind the growing tip. (*C*) Higher magnification of an intact root cap. Marker = 150 μm.

Table 1. Hyphal branching of germinated *Gigaspora gigantea* spores induced by root caps isolated from Ri T-DNA transformed carrot roots grown in liquid culture. Isolated root caps were suspended in sterile water and stored in a refrigerator for 0–5 d. After storage, the root cap suspension was vortexed, centrifuged for 3 min at 3500 rpm, and the supernatant was assayed. The pelleted root caps were then resuspended and the suspension (root caps) was bioassayed. Control hyphae were treated with sterile water. 40 µl of sample was applied to each unbranched hypha tested.

	Branches induced after days of storage of root caps at 4 °C[a]			
	Day 0	Day 1	Day 5	Day 5 after washing[b]
Control	0.60 ± 0.22			
Supernatant	0.40 ± 0.16	3.20 ± 0.42	6.50 ± 0.78	0.40 ± 0.16
Root caps	8.80 ± 0.42	8.50 ± 0.58	10.20 ± 0.65	8.50 ± 0.50

[a] Branches induced ± SEM, $n = 10$.

[b] After testing the samples on day 5, the pelleted root caps were drained, washed one time with sterile water, and resuspended in the same volume as before centrifugation. The supernatant and suspension were then assayed again.

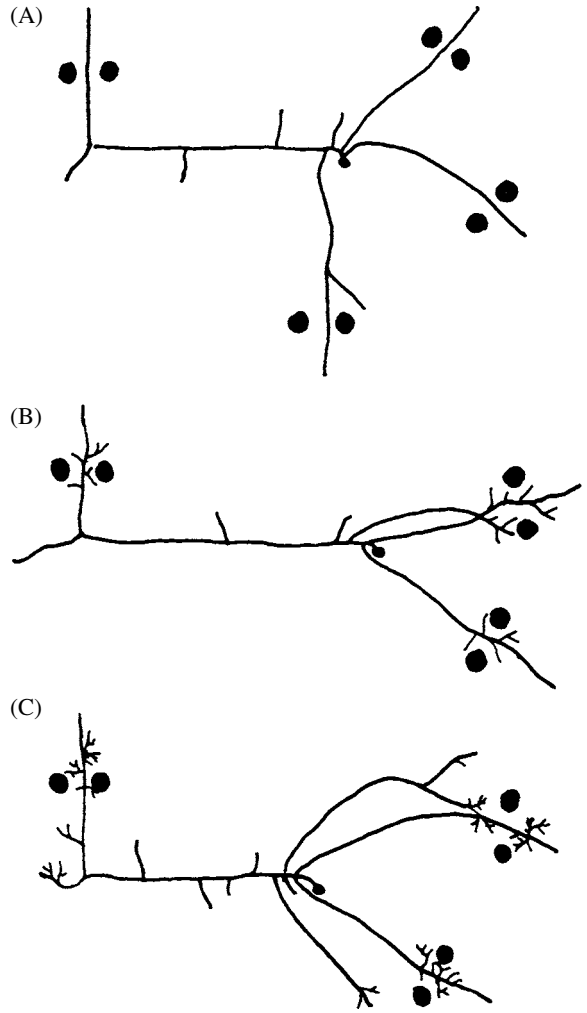

Fig. 3. Tracings of hyphal branches of *Gigaspora gigantea* induced by isolated root caps and a slow sedimenting particulate fraction (SSPF) from carrot roots grown in culture. (*A*) Control bioassay injected with sterile water. (*B*) Bioassay with SSPF. (*C*) Bioassay with isolated root caps. Tracings were made right on the Petri dish (actual size) to show an overview of the effects of two isolated particulate fractions on the growth of germinated spores. Black dots represent the holes in which the samples were applied.

potent stimulator (Fig. 7). The specific branching activities for the carrot root caps (10 branches 39.2 µg D.W.$^{-1}$ = 0.25 branches µg^{-1}) and carrot root exudate (10 branches 12 ng^{-1} = 833 branches µg^{-1}) were directly comparable. The specific branching activity of the corn root BC was similar to those of carrot root caps (10 branches 55.2 µg^{-1} = 0.18 branches µg^{-1}).

DISCUSSION

The first stage of interaction between an AM fungus and host root appears to be a hyphal growth and branching response of the fungus to constitutive components (Giovannetti *et al.* 1993) secreted in the host root exudates. It has been proposed that AM fungi can sense a concentration gradient of host generated chemicals which are most concentrated at or near the surface of the root (Giovannetti *et al.* 1993, Nagahashi & Douds 2000). Most of the work on the pre-infection stage has focused on the soluble and volatile components of the exudate (Graham 1982, Bécard & Piché 1989, Nair *et al.* 1991, Giovannetti *et al.* 1993, Nagahashi & Douds 2000) although one report suggested root 'mucilages' induced differential hyphal morphogenesis of an AM fungus (Giovannetti *et al.* 1993). The soluble exudates have been studied the most since they have been shown to contain the greatest amount of hyphal branching stimulators (Nagahashi & Douds 1999). However, it is known that roots can be infected and colonized along the axis of the root (Smith, Dickson & Walker 1992, Giovannetti *et al.* 1996). Since most of the root exudation occurs in the first 1–2 mm of the root apex (Griffin, Hale & Shay 1976, Curl & Truelove 1986, Zhao, Schmitt & Hawes 2000) and experiments have shown that certain root exudates stay very close to the site or sites of exudation (Curl & Truelove 1986), it is possible that other sources of hyphal branching stimulators are present along the axis of the root. The results reported here showed that particulate fractions such as root caps, fragments of

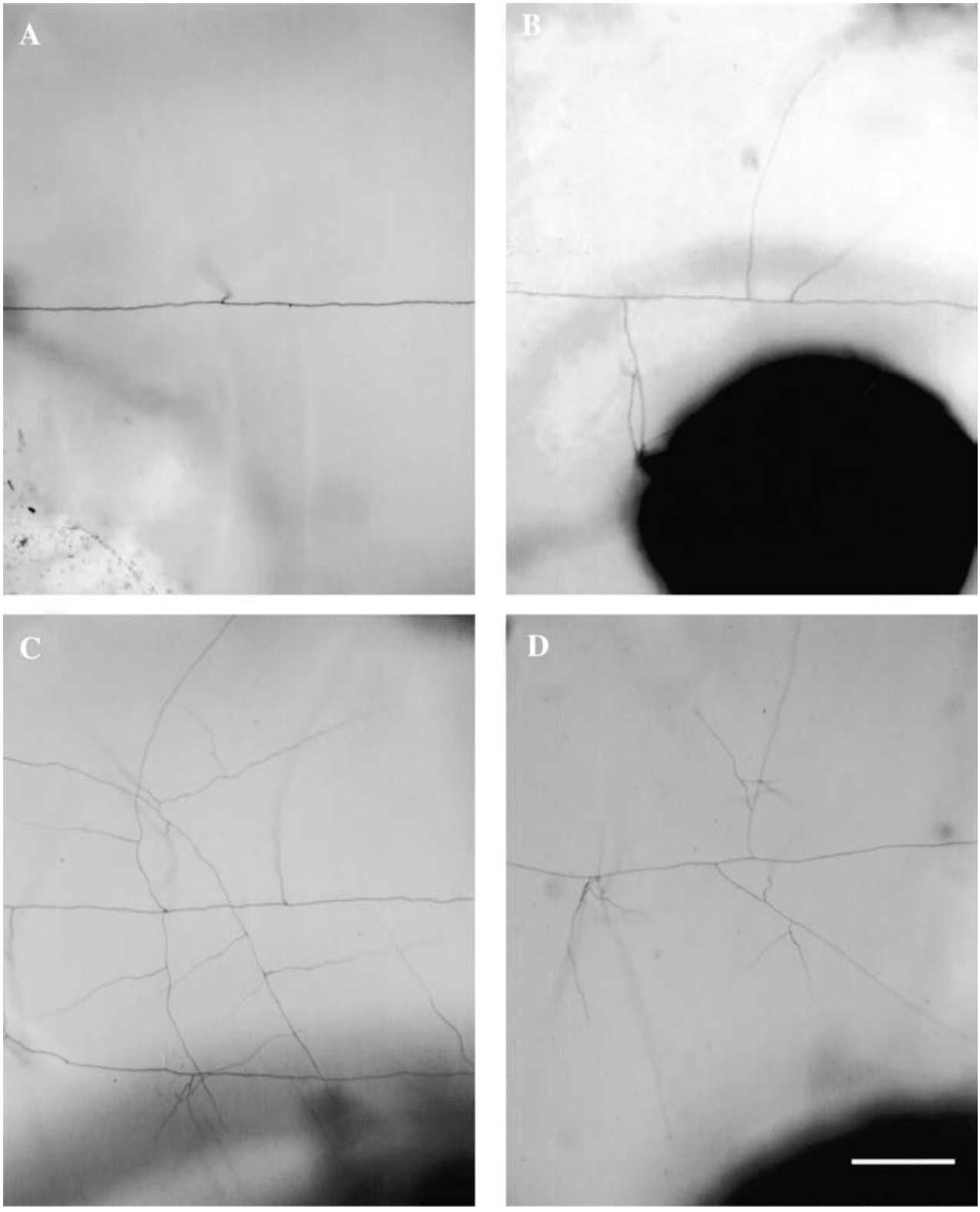

Fig. 4. Actual micrographs of hyphal branches of *Gigaspora gigantea* induced by root caps and the slow sedimenting particulate fraction (SSPF) isolated from carrot root cultures. (*A*) Holes of the control were injected with water near a primary germ tube. (*B*) Holes injected with SSPF near a primary germ tube. (*C*) Holes injected with isolated root caps near a primary germ tube. The long straight hypha going across the middle of the micrograph is the primary germ tube. The bottom long curving hypha is secondary. (*D*) Holes injected with isolated root caps near a major secondary hypha. The plates were stained with trypan blue and frequently the holes filled with particles of stain and remained dark. The light circular area in the lower left of A and the dark circular areas in B, C, and D are the holes in which the samples were applied. Bar = 1 mm.

Table 2. Hyphal branches of germinated *Gigaspora gigantea* spores induced by root caps and a slow sedimenting particulate fraction (SSPF) isolated from carrot roots grown in liquid culture in M medium minus phosphorus.

	Number of branches induced[a]
Control[b]	0.60 ± 0.22
SSPF	6.10 ± 0.32
Root caps	12.20 ± 1.41

[a] Branches induced ± SEM, $n = 8$.
[b] Control was treated with 40 µl of sterile water and experimental with 40 µl of SSPF or root caps.

Table 3. Hyphal branches of germinated *Gigaspora gigantea* spores induced by border cells and mucilage isolated from aseptically grown corn roots.[a]

	Number of branches induced[b]
Control	0.75 ± 0.30
Mucilage + Border Cells[H]	15.38 ± 2.40
Mucilage	2.88 ± 0.41

[a] Experimental tested with 40 µl of mucilage + BCs before filtering or 40 µl of mucilage after filtering to remove BCs.
[b] Branches induced ± SEM, $n = 8$.

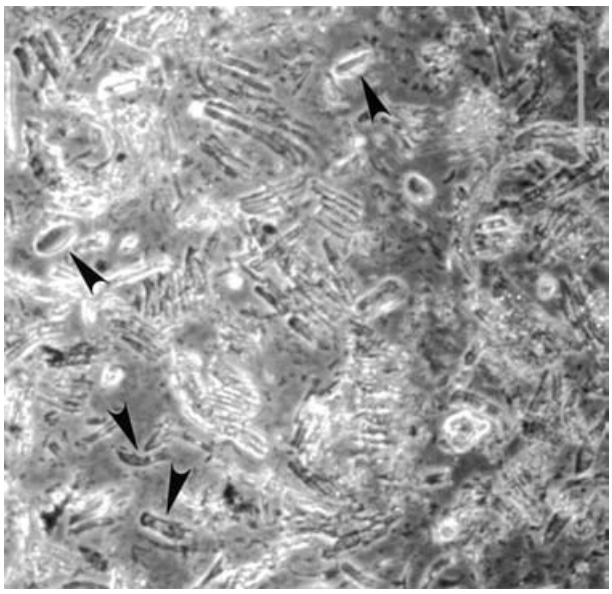

Fig. 5. Phase contrast photomicrographs of the slow sedimenting particulate fraction isolated from transformed carrot roots grown in liquid culture. The micrograph was taken after treatment with 10 mM citrate buffer at pH 6.0 which was used to dissolve small pieces of gellan. Although small pieces of root caps dominate this fraction, some individual border cells can be seen (arrows). Marker = 150 µm.

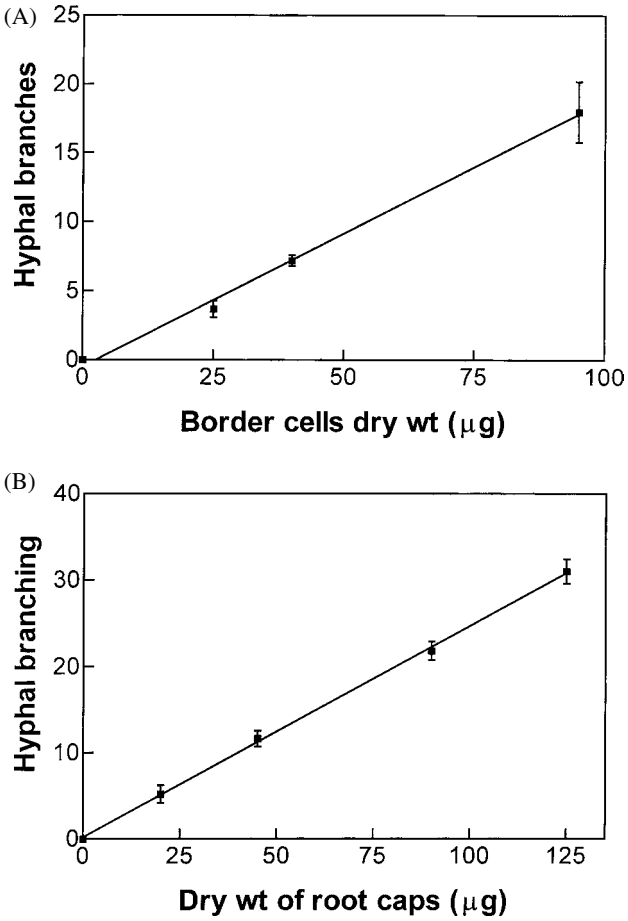

Fig. 6. (*A*) Relationship between the dry weight of isolated border cells from corn roots and the number of hyphal branches induced on germinated *Gigaspora gigantea* spores. (*B*) Relationship between the dry weight of isolated root caps from carrot roots and the number of hyphal branches induced on germinated spores. Means of five observations ± SEM.

root caps, BCs, and mucilage all contain compounds which stimulate hyphal branching of an AM fungus. Micrographs of carrot roots grown in culture on solid medium (Fig. 2) show that root caps and fragments of root caps can adhere to or are found very close to the root surface at considerable distances from the growing tip. Border cells have also been shown to occur on or near the surface of roots at considerable distances from the root apex (Hawes *et al.* 2003).

The fact that isolated root caps from P stressed roots (Table 2) had more branching stimulating activity than roots caps from the control (Table 1) was not a surprise. Growth of onion (Tawaraya *et al.* 1996) or carrot roots (Nagahashi & Douds 2000) under minus P conditions has been shown to increase hydrophobic compounds in root exudates that stimulate hyphal growth and branching. These hydrophobic compounds, generated under P stress, have also further stimulated appressorium formation and colonization of onion roots (Tawaraya, Hashimoto & Wagatsuma 1998).

Whether or not the particulate fractions are a source or site of synthesis of hyphal branching stimulators, remains to be determined. It may be that the branching stimulators associated with particulate fractions (Table 1) are simply exudate components which adhere to root caps and fragments of root caps during isolation procedures. If this latter case is true, then these

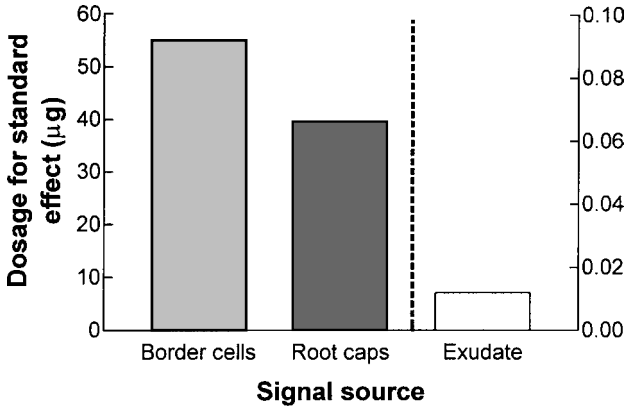

Fig. 7. The dry weight (dose) of various isolated fractions that were necessary to induce a standard effect of 10 hyphal branches. The root caps and exudate were isolated from transformed carrot roots grown in liquid culture and can be directly compared. The dry weight of border cells from corn roots are shown for comparison.

compounds must be tightly associated with the cell walls of root caps since they were washed thoroughly before testing in the original bioassay. These compounds could conceivably be released with other cell wall constituents as a result of autolysis of cell walls (Sasaki *et al.* 1987) during storage. Alternatively, they could be synthesized by root caps as suggested by Table 1 but this would imply that the root caps are metabolically active under our storage conditions. For BCs, it is possible that they are uniquely synthesized compounds which are not found in the root meristem or adjacent cells (Brigham *et al.* 1995). Whether or not the branching stimulator found in the mucilage is actually exuded by the BC into the mucilage, needs to be determined. To date, no naturally occurring hyphal branch stimulator has been identified so the uniqueness or similarity of soluble exudate compounds compared to those of particulate root components still needs to be resolved.

Hyphal branching stimulators (regardless of type or source) increase the chances of an AM fungal hyphal tip making contact with an epidermal cell surface site and thereby allowing appressoria formation and eventual colonization of the root to occur. Particulate sources of hyphal branching stimulators may not appear to be significant relative to dry weight of the sample when compared to the soluble exudates (*cfr* specific activities in Fig. 7). However, they are particulate and therefore would not readily diffuse away from the root surface and can be seen adhering to the root surface at great distances from the root tip (Fig. 2). The presence of these particulate sources of fungal branching stimulators can help explain why host roots can be infected anywhere along the root axis. Infection can occur in immature as well as mature regions of the root behind the apical tip (Smith *et al.* 1992) and stimulation of differential hyphal morphogenesis can occur along the entire root system (Giovannetti *et al.* 1996).

Finally, the results reported here show that isolated border cells contain an AM fungus hyphal branching stimulator and therefore these cells may play a role in the precolonization events of endomycorrhizal fungi. This result is consistent with the correlation which showed that those plant families that readily generate border cells are also more likely to be colonized by AM fungi (Niemera *et al.* 1996) than plants that do not readily release border cells.

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